## 1. PURPOSE

1.1 This document outlines the procedure to obtain peripheral blood mononuclear cells (PBMC) and plasma from human whole blood for use in cell activation/Cell Mediated Immunity (CMI) and humoral immunity/serology studies.

## 2. SCOPE

2.1 See policy (DMID-LB-POL-00001) for scope of this SOP.

## 3. DEFINITIONS

3.1 **Aliquot**: Equal fractions of the entire specimen.

For additional definitions, see DMID glossary.

## 4. RESPONSIBILITIES

4.1 Clinical Site Principal Investigator is responsible for oversight of processing and communicating deviations to DMID.

4.2 Laboratory directors/ managers, or other assigned site personnel, are responsible for training and ensuring adequate documentation of the training in accordance with institutional requirements and GCLP. Samples must be processed according to institutional Biosafety Guidelines and in accordance with all applicable safety procedures including universal precautions for blood-borne pathogens. Any deviation from the SOP should be recorded. This documentation will be stored indefinitely or per DMID guidelines.

4.3 Laboratory personnel are responsible for following the SOP and documenting the procedure. Processing must be performed using strict aseptic procedures and performed in a Class II Biological Safety Cabinet (BSC). Ensure reagents, supplies and equipment are available and appropriate per the appendices (see appendix A, B, and C).

## 5. PROCEDURE

### INITIAL PROCESSING AND CELL ISOLATION

USE ONLY ONE OF THE FOLLOWING THREE CELL ISOLATION METHODS:

- Cell Isolation and Plasma Collection for Blood Collected in sodium citrate CPT tubes (See 5.1)
- Cell Isolation and Plasma Collection for Blood Collected in ACD, NaHep, or EDTA tubes with pre-filled Frit Barrier (See 5.2)
- Cell Separation by Manual Density Gradient Media Overlay or Underlay (Ficoll Separation) (See 5.3)

Note on timing: The entire process (from blood collection to freezing down cells) should be completed within eight hours.

Carefully check the participant identification number on all tubes of blood received. Organize primary tubes such that there is no possibility of mixing tubes among different participants.

Suggestion: Place all tubes for each participant/anticoagulant in one rack. Different racks can be used to separate participants or tube types, and a different color marker can be used for each participant to avoid confusion.

Determine and record an accurate measurement of the usable whole blood volume within 0.5mL. The volume of usable whole blood is not necessarily equal to the tube size.

5.1 Cell Isolation and Plasma Collection for Blood Collected in sodium citrate CPT tubes

Note on timing: Centrifuge tubes as soon as possible but no later than four hours after blood collection. Record and report the time when centrifugation starts.

5.1.1 Store plasma collection tubes in an upright position at room temperature (15°C to 30°C) until centrifugation.

5.1.2 Gently invert the tubes eight to ten times to remix cells immediately before centrifugation.

5.1.3 Centrifuge tubes at room temperature (15°C to 30°C) in a horizontal rotor (swing-out head). Centrifuge for 30 minutes at 1800 x g (RCF). RCF must be calculated carefully (see the equation below). Do not use brake. Care should be taken to ensure that CPT tubes are properly seated in the centrifuge insert/bucket.

$$RCF = \left(\frac{RPM}{1{,}000}\right)^2 \times r \times 1.118 \quad \Rightarrow \quad RPM = \sqrt{\frac{RCF}{r \times 1.118}} \times 1{,}000$$

RCF = Relative Centrifugal Force

RPM = Rotational Speed (revolutions per minute)

$r$ = centrifugal radius in mm = distance from the center of the turning axis to the bottom of the centrifuge

5.1.4 After the centrifuge has come to a complete stop, carefully remove the tubes and place in a rack.

The mononuclear cells and platelets will be in a whitish layer just under the plasma layer (see the figure below).


*The picture was provided by the IVQAC Laboratory, Duke Human Vaccine Institute.*

5.1.5 Aspirate the plasma from each of the CPT tubes without disturbing the cell layer. If the protocol requires plasma to be collected, please refer to protocol-specific documents (e.g., the Manual of Procedures [MOP]) for instructions on the number, volume, any special handling of the aliquots, and the storage temperature.

5.1.6 Collect the mononuclear cell layer from each of the tubes with a pipette and transfer them to a 50mL plastic conical centrifuge tube with cap.

5.1.7 STRONGLY RECOMMENDED additional step to maximize recovery of mononuclear cells: Following collection of the mononuclear cells in step 5.1.6, CPTs may be gently rinsed to maximize recovery of the mononuclear cells. Following initial PBMC harvest, slowly add 1 mL of PBS down the side of each CPT; gently rinse the Polyester Gel and then add the rinse to the 50 mL centrifuge tube following the guidance outlined in steps 5.1.8 and 5.1.9.

5.1.8 If three CPT tubes or fewer (≤3) are collected, place the cell layers collected from all the CPT tubes into one 50mL centrifuge tube.

5.1.9 If more than three CPT tubes (>3) are collected, place the cell layers of two to three of the CPT tubes into one 50mL centrifuge tube. Do not combine the cell layers of more than three CPT tubes into one 50mL centrifuge tube. Repeat this step until the cell layers from all the CPT tubes are transferred to 50mL centrifuge tubes, then proceed with the wash steps below.

5.1.10 Proceed to Section 5.4 for Washing.

5.2 Cell Isolation and Plasma Collection for Blood Collected in ACD, NaHep, or EDTA tubes with pre-filled Frit Barrier

Note on timing: Optimum processing time for PBMC is less than four hours from the time of adding blood to the cell separation tubes (ACCUSPIN or equivalent) to the initiation of the controlled-rate freezing cycle.

Ensure that the tubes are at room temperature before processing.

Before adding the blood, visually check the CSTFB to see if there is liquid above the frit. If there is liquid above the frit, centrifuge the CSTFB at 1000 x g for 30 seconds. If any density gradient solution remains above the frit after centrifuging, it should be aspirated.

5.2.1 Blood Dilution for CSTFB separation

The maximum ratio of blood to Saline Solution should be approximately 2:1. Use one 50mL tube for each 10 to 20mL of whole blood (or one 12 to 14mL tube for each 5 to 10mL of whole blood). Use as many CSTFB as required to distribute all of the blood for each participant.

Note on timing: Density gradient media is toxic to cells; work quickly and efficiently during the separation steps.

5.2.1.1 Label each CSTFB with the participant identification number.

5.2.1.2 Using a sterile pipet, add Saline Solution to each CSTFB:

| **CSTFB Size (mL)** | **Approximate Volume of Saline Solution (mL)** |
|---|---|
| 50 | 5 |
| 15 | 2 |

5.2.1.3 Mix whole blood gently, then use a sterile pipet to transfer blood into the labeled CSTFB.

| **CSTFB Size (mL)** | **Approximate Volume of Blood (mL)*** |
|---|---|
| 50 | 10 to 20 |
| 15 | 4 to 5 |

**Note: Lower blood volumes, especially in the presence of low hematocrits, may cause the buffy coat to drop close to/onto the frit, making it difficult to harvest. Higher blood volumes may contribute to increased background/debris in specimens. Refer to protocol-specific guidelines for lower blood draw volumes.*

5.2.1.4 Using a sterile pipet, rinse each original anticoagulated blood tube with Saline Solution and transfer rinse volumes to the CSTFB, making sure not to exceed the total tube volume (Saline Solution + Whole Blood) limit.

| **CSTFB Size (mL)** | **Total Tube Volume Limit (mL) (Whole Blood + Saline Solution)** |
|---|---|
| 50 | 30 |
| 15 | 7.5 |

5.2.1.5 Carefully cap the CSTFB.

5.2.2 CSTFB Density Centrifugation and PBMC Collection

5.2.2.1 Hold the tubes in an upright position and gently transfer them to the centrifuge.

5.2.2.2 Centrifuge at 800 to 1000 x g for 15 minutes at 15°C to 30°C with the brake off.

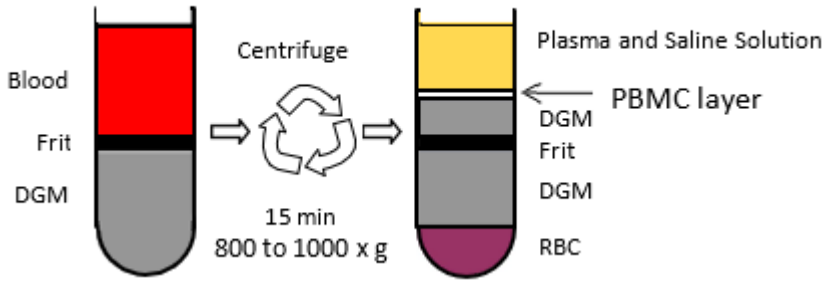
- PBMC separation may be improved for some specimens by centrifuging at 1000 x g. If the brake is on, it will disrupt the layers.

5.2.2.3 While the tubes are centrifuging, prepare new sterile conical centrifuge tubes to be used in step 5.2.2.9; this will be the same number of tubes used in step 5.2.2.2 above. Label each tube with the participant identification number.

5.2.2.4 Gently remove the CSTFB from the centrifuge so as not to disturb the layers.

5.2.2.5 Centrifugation results in the tube's contents dividing into six distinct layers including the frit. From the top of the tube, these are:

- Plasma and Saline Solution
- PBMC layer
- Density gradient media (DGM)
- Frit
- Density gradient media
- Packed red blood cells (RBC) and granulocytes




5.2.2.6 Inspect the tubes for the following possible problems. Document observations and any follow-up actions taken according to network and laboratory requirements.

- Hemolysis in the Plasma + Saline Solution layer.
- Clots visible on the frit after centrifugation.
- Poor PBMC layer due to error in centrifugation such as speed, time, or braking. PBMC layer will appear small and indistinct, while the Plasma + Saline Solution layer may be slightly cloudy.
- PBMC layer formed on frit due to low RBC count or hematocrit volume.

5.2.2.7 Using a new sterile pipet (serological or transfer pipet) for each participant, remove the upper yellowish Plasma + Saline Solution fraction down to within approximately 1 to 2cm of the cloudy white PBMC band located at the interface between the Plasma + Saline Solution (yellowish) fraction and the clear separation medium solution. Discard the Plasma + Saline Solution fraction per laboratory policy.

- Alternatively, the upper Plasma + Saline Solution fraction may be left in place and the cloudy white PBMC band may be removed by carefully inserting the pipet through the upper layer to the PBMC band.

5.2.2.8 Using a sterile serological pipet, collect all cells at the cloudy white interface above the frit. Take care not to aspirate any more separation medium solution than necessary.

5.2.2.9 Transfer the collected cells from one CSTFB to a single corresponding, pre-labeled, sterile conical centrifuge tube prepared in step 5.2.2.3. Tubes can be pre-filled with Saline Solution to save time.

| **CSTFB Size (mL)** | **Conical Centrifuge Tube Size (mL)** | **Saline Solution Pre-Fill Volume (mL)** |
|---|---|---|
| 50 | 50 | 25 |
| 15 | 15 | 5 |

5.2.2.10 Re-cap the CSTFB containing the remaining red blood cells and separation media. Discard the CSTFB as biohazard waste following laboratory policy.

5.2.2.11 Proceed to Section 5.4 for Washing.

5.3 Cell Separation by Manual Density Gradient Media Overlay or Underlay and Blood Dilution (Ficoll Separation)

5.3.1 Blood Dilution

5.3.1.1 Uncap the tubes of anticoagulated blood.

5.3.1.2 Label each conical centrifuge tube with the participant identification number.

| **Conical Centrifuge Tube Size (mL)** | **Approximate Blood Volume (mL)** |
|---|---|
| 50 | 12 to 22 |
| 15 | 4 to 5 |

5.3.1.3 Transfer the blood to a sterile, labeled 15 or 50mL conical centrifuge tube and add sufficient volume of Saline Solution to dilute the blood according to the density gradient media package insert (maximum ratio of blood to diluent should be 2:1).

5.3.2 Density Gradient Cell Separation

On any given sample, use either the Overlay Method (5.3.2.1) or the Underlay Method (5.3.2.2), but not both methods

5.3.2.1 Overlay Method

5.3.2.1.1 Prepare a sterile, labeled conical centrifuge tube for each tube containing diluted blood.

5.3.2.1.2 Aseptically add the appropriate volume of density gradient media to the empty sterile conical centrifuge tubes. The volume of density gradient media will depend on the ratio of density gradient media to diluted blood recommended by the manufacturer.

5.3.2.1.3 Carefully and slowly pipet diluted blood <u>on top</u> of the density gradient media. Gently allow the Saline Solution-diluted blood mixture to flow down the side of the tube and pool on top of the density gradient media surface without breaking the surface plane; slowly pipetting the blood mixture at a constant speed facilitates the layering in this step.

5.3.2.1.4 Carefully cap the tubes. Proceed to step 5.3.3.

5.3.2.2 **Underlay** Method

5.3.2.2.1 Prepare a sterile, labeled conical centrifuge tube for each tube containing diluted blood.

5.3.2.2.2 Aseptically add the appropriate volume of density gradient media to the empty sterile conical centrifuge tubes. The volume of density gradient media will depend on the ratio of density gradient media to diluted blood recommended by the manufacturer.

5.3.2.2.3 Carefully and slowly pipet density gradient media solution <u>under</u> blood- Saline Solution.

5.3.2.2.4 Carefully cap the tubes. Proceed to step 5.3.3.

5.3.3 Lymphocyte Density Centrifugation and PBMC Collection

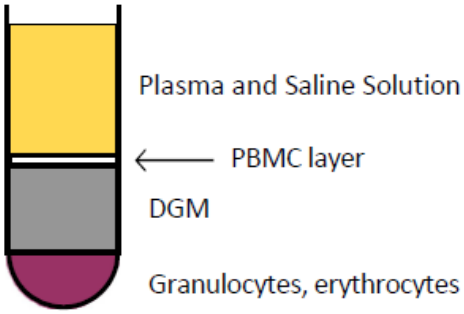
5.3.3.1 Hold the tubes in an upright position and gently transfer them to the centrifuge.

5.3.3.2 Centrifuge at 400 x g for 30 minutes at 15°C to 30°C with the <u>brake off</u>, as outlined in the package insert that accompanies the gradient media.

- If the brake is on, it will disrupt the layers. The centrifuge brake must be turned <u>off</u> for the separation to be clean and to maximize retrieval of the PBMCs.

5.3.3.3 Centrifugation results in the tube's contents dividing into four distinct layers. From the top of the tube, these are:

- Plasma and Saline Solution
- PBMC layer
- Density gradient media (DGM)
- Granulocytes, erythrocytes



5.3.3.4 While the tubes are centrifuging, prepare new sterile conical centrifuge tubes to be used in step 5.3.3.9; this will be the same number of tubes used in step 5.3.3.2. above. Label each tube with the participant identification number.

5.3.3.5 Remove the tubes from the centrifuge.

5.3.3.6 If the cell layer is not visible, confirm that the centrifuge is operating properly. Correct any problems you find. Re-centrifuge the tubes. Document the problem and actions taken in study records.

- If the cell layer is still not visible after re-centrifuging, document, remove and discard the Saline Solution supernatant, and proceed.
- If the plasma is very cloudy, it may be difficult to see the interface with the density gradient media. It is possible to improve the collection of lymphocytes by removing most of the plasma above the interface with a 10mL pipet, leaving only 0.5cm remaining. This allows for better positioning of the tip of the pipet for collection of cells.

5.3.3.7 Using a new sterile serological pipet for each participant, remove the upper yellowish Plasma + Saline Solution fraction down to within approximately 1 to 2cm of the cloudy white PBMC band located at the interface between the Plasma + Saline Solution (yellowish) fraction and the clear separation medium solution. Discard the Plasma + Saline Solution fraction per laboratory policy.

- Alternatively, the upper Plasma + Saline Solution fraction may be left in place and the cloudy white PBMC band may be removed by carefully inserting the pipet through the upper layer to the PBMC band.

5.3.3.8 Using a sterile serological or transfer pipet, collect all cells at the cloudy white interface. Take care not to aspirate any more separation medium solution than necessary.

5.3.3.9 Transfer the collected cells from one conical centrifuge tube to a single corresponding, pre-labeled, sterile conical centrifuge tube prepared in step 5.3.3.4. Tubes can be pre-filled with Saline Solution to save time.

| **Conical Centrifuge Tube Size (mL)** | **Saline Solution Pre-Fill Volume (mL)** |
|---|---|
| 50 | 25 |
| 15 | 5 |

5.3.3.10 Re-cap the conical centrifuge tube containing the remaining red blood cells/separation medium and discard the tube as biohazard waste following laboratory policy.

5.3.3.11 Proceed to Section 5.4 for Washing.

5.4 Washing, Counting, Resuspension, Concentration, Overnight Controlled-Rate Freezing, Storage, and Shipment

5.4.1 Wash #1:

5.4.1.1 Bring up to the recommended volume of the PBMC fraction by adding Saline Solution.

| Tube Size (mL) | Recommended Volume (mL) |
|---|---|
| 50 | 45 |
| 15 | 10 |

5.4.1.2 Re-cap all of the harvested cell tubes. Mix gently by inverting five times.

5.4.1.3 Centrifuge at room temperature (15°C to 30°C) for ten minutes at 300 x g (RCF) (allowable range 200-400g). Low brake may be used. Remove as much supernatant as possible without disturbing the cell pellet by quickly decanting or "shock dumping" into the designated waste container in the Biological Safety Cabinet.

5.4.2 Wash #2:

5.4.2.1 Resuspend the cell pellet by gently tapping the tube with index finger.

| Tube Size (mL) | Saline Solution Resuspension Volume (mL) |
|---|---|
| 50 | ≤ 5 |
| 15 | ≤ 3 |

5.4.2.2 Combine the cell suspensions from the same participant identification number, time, and date of draw. This is the harvested cell tube.

| Tube Size (mL) | Number of Pellet Suspensions to Combine | Total Volume (mL) |
|---|---|---|
| 50 | ≤ 4 | ≤ 20 |
| 15 | ≤ 2 | ≤ 6 |

5.4.2.3 Use a small volume of Saline Solution to rinse the tubes from which the pellets were transferred. Collect the Saline Solution rinse in the harvested cell tube.

5.4.2.4 Bring up to the recommend volume of the PBMC fraction by adding Saline Solution.

| Tube Size (mL) | Recommended Volume (mL) |
|---|---|
| 50 | 45 |
| 15 | 10 |

5.4.2.5 Re-cap the tubes and place the tubes in the centrifuge.

5.4.2.6 Centrifuge at room temperature (15°C to 30°C) for ten minutes at 300 x g (RCF) (allowable range 200-400g). Low brake may be used. Remove as much supernatant as possible without disturbing the cell pellet.

5.4.3 PBMC Cell Count

5.4.3.1 Add Saline Solution equal to approximately 20% of the collected blood volume in each 50mL centrifuge tube to resuspend the cell pellet. Record this volume (V). Mix cells by gently tapping the tube with index finger.

Note: While V is usually approximately 20% of the usable whole blood volume rounded to the nearest mL, V may vary depending on the size of the cell pellet and the cell counting method.

5.4.3.2 Count cells per the lab's cell counting SOP. Record and report this as the Total Cell Count.

5.4.3.3 Determine the volume (V1) of CPS (10% DMSO in FBS) to add with serological pipets to adjust the concentration to approximately, but not less than, $5x10^6$ cells/mL using the following formula:

$$V1 = (T/N1) \times V2$$

T = Total cell count

N1 = Target final cell concentration ($5x10^6$ cells/mL or as provided in MOP or other study- specific documentation)

V2 = final aliquot volume in mL (typically 1mL/cryovial, refer to study-specific materials)

5.4.4 Final Centrifugation

5.4.4.1 Bring up to the recommended volume of the PBMC fraction by adding Saline Solution.

| **Tube Size (mL)** | **Recommended Volume (mL)** |
|---|---|
| 50 | 45 |
| 15 | 10 |

5.4.4.2 Centrifuge at room temperature (15°C to 30°C) for ten minutes at 300 x g (RCF) (allowable range 200-400g). Low brake may be used. Remove as much supernatant as possible without disturbing the cell pellet.

5.4.4.3 Calculate the number of cryovials need to be labeled to store the entire PBMC specimen. Verify that all cryovials are labeled, in the correct sequential order, and easily accessible so that the following steps can be completed efficiently.

Note: Do not discard any of the PBMC specimen. There is no maximum number of aliquots.

5.4.5 Aliquoting for Cryopreservation

Note on timing: The following steps should be performed quickly to preserve cell integrity.

5.4.5.1 Gently resuspend the cell pellet by flicking, racking, or pipetting.

5.4.5.2 Gently add CPS to the re-suspended cells with continuous swirling, using the volume of cold CPS (V1) that you determined in 5.4.3.3.

5.4.5.3 Work quickly once the CPS has been added. Do not allow the cells to sit in the CPS for longer than ten minutes before placing in the freezer.

5.4.5.4 Aliquot 1mL per cryovial, unless directed to store at a different volume by the protocol- specific MOP.
Note: If less than 1mL of PBMC suspension remains after aliquoting the calculated number of vials to be stored, distribute the excess volume equally across the other PBMC vials.

5.4.6 Overnight Controlled-Rate Freezing

5.4.6.1 Immediately transfer all cryovials to the controlled-rate freezing container.

5.4.6.2 Mr. Frosty and CoolCell: Close the container and place it in a -80°C freezer (-70°C to -95°C), in a location that is not disturbed by repeated freezer access (i.e., away from the front or top of the freezer near the opening door/lid) for a minimum of four hours.

5.4.6.3 StrataCooler: Close the container and place it in a -80°C freezer (-70°C to -95°C), in a location that is not disturbed by repeated freezer access (i.e., away from the front or top of the freezer near the opening door/lid) for a minimum of overnight.

5.4.7 Storage and Shipment

5.4.7.1 Sample storage/shipment requirements should be detailed in the protocol-specific MOP. Unless a waiver is granted by the OCRR Director, all PBMCs collected under DMID-contract funded protocols must be shipped to the DMID Clinical Materials Services (CMS) for central storage.

5.4.7.2 If the cells will be stored in the freezer (-70°C to -95°C) at the site per the study-specific MOP, the cells must be shipped on dry ice to the network-specific repository/central storage within two weeks of collection.

5.4.7.3 If the cells will be stored at liquid nitrogen (LN2) at the site per the study-specific MOP, the cells must be moved from the freezer (-70°C to -95°C) to LN2 the next day; subsequently the cells must always be shipped at LN2 to the network-specific repository/central storage.

## 6. REFERENCES

6.1 DMID-LB-POL-00001-Requirements for Use of DMID PBMC Processing SOP

## 7. APPENDICES

7.1 **APPENDIX A: REAGENTS**

7.2 **APPENDIX B: EQUIPMENT AND MATERIALS**

7.3 **APPENDIX C: REAGENT PREPARATION**

## 8. REVISION HISTORY

8.1 DMID-LB-SOP-00001 revision 01 was rewritten in part from prior version-OCRR SOP 002. This SOP is original version within the eQMS.

## 9. ADDITIONAL INFORMATION

9.1 Document Lead: OCRR

9.2 Posting externally: yes

**APPENDIX A: REAGENTS**

**(NOTE: EQUIVALENTS MAY BE USED, UNLESS NOTED OTHERWISE, AND SHOULD BE DOCUMENTED)**

1.0 Saline Solution

1.0.1 A single balanced salt solution (PBS or HBSS) should be used by a site throughout the life of the protocol.

- Saline Solution can be stored either at room temperature (15°C to 30°C) or refrigerator temperature (2°C to 8°C).
- After opening, keep the opened bottle of Saline Solution sterile and use the contents within 30 days or the original manufacturer's expiration date, whichever comes earlier.

1.0.2 $Ca^{+2}$, $Mg^{+2}$-free phosphate buffered saline (PBS) (Fisher Scientific, Cat. # SH30256.02)

1.0.3 Alternative: Hanks' Balanced Salt Solution (HBSS) without calcium or magnesium, ready- to-use (Fisher Scientific, Cat. 14-175-095)

1.1 Fetal Bovine Serum (FBS), Heat Inactivated (HI) (R&D Systems, Inc., Cat. #S12450H)

1.1.1 Prior to use, FBS should be stored at -5°C to -20°C (per the manufacturer's recommendation) in its original container and can be used until the manufacturer's expiration date. Once thawed, FBS can be stored at 2°C to 8°C and is stable for one month or the original manufacturer's expiration date, whichever comes earlier.

1.1.2 Aliquot into sterile, labeled 50mL conical centrifuge tubes or other size aliquots appropriate for the anticipated workload. Labels should identify these tubes as "HI-FBS" and include the lot number, aliquot date, expiration date, and technician's initials. Aliquots of FBS should be stored frozen (per the manufacturer's recommendation) and are stable until the manufacturer's original expiration date.

1.1.3 The same lot number of FBS should be used through the lifecycle of the protocol.

1.1.4 In the event the recommended product is not available, prior to use of an equivalent product for per-protocol processing the lot should be tested for LPBS content.

1.2 Dimethyl sulfoxide (DMSO) (cell-culture grade; Sigma-Aldrich, Cat. #D2650)

1.2.1 Store unopened bottles at room temperature (15°C to 30°C). Check bottle for expiration date and discard if expired.

1.2.2 After opening, undiluted DMSO is stable at room temperature (15°C to 30°C) when protected from light and moisture for six months or the labeled expiration date, whichever comes earlier.

1.2.3 Discard open bottle if visible signs of contamination are noted.

1.2.4 Reagent may be aliquoted in small amounts to help preserve sterility. Label aliquots with "DMSO," the date opened/aliquoted, the expiration date (six months from opening), and technician's initials. Protect aliquots from light.

1.3 Isopropanol (if using Mr. Frosty Freezing containers)

1.4 Cell Counting Reagents

1.4.1 The requirements for counting reagents will vary depending on the method used.

1.4.2 0.4% trypan blue solution (an example of a common cell viability staining solution for cell counting)

**APPENDIX B: EQUIPMENT AND MATERIALS**

Note: Equivalents may be used. If an equivalent is used, it should be documented.

1.0 Blood collection tubes as defined in study-specific documentation. Options include:

- 8mL Cell Preparation Tubes (CPT) with sodium citrate (BD, Cat. #362761)
- ACD, NaHep, EDTA blood collection tubes

1.1 Cell Separation Tube with Frit Barrier (CSTFB). If CPT is used, then CSTFB is not applicable.

A single method (CPT, CSTFB, or a manual overlay/underlay with a conical centrifuge tube) for cell separation should be indicated in study-specific processing instructions.

1.1.1 Pre-filled CSTFB (1.077 density gradient media)

- Examples: Leucosep pre-filled CSTFB with 1.077 density gradient media (Greiner Bio- One, Cat. #227288, 50mL capacity) or ACCUSPIN™ System Histopaque® - 1077 (Sigma-Aldrich, Cat. #A2055, 50mL capacity)
- Note: The capacity of the tube required will depend on the whole blood volume.
- Storage conditions:
  - Store in the refrigerator (2°C to 8 C)
  - Protect from light
  - A cloudy appearance indicates deterioration of the product.
  - Allow CSTFB to come to room temperature (15°C to 30°C) prior to use.

1.1.2 Alternatives to pre-filled CSTFB system:

- Dry CSTFB; for example, Leucosep separation tubes (Greiner Bio-One, Cat. #227290, 50mL capacity), combined with density gradient media (below). Follow manufacturer's instructions for storing and setting up tubes.
- 1.077 density gradient media; for example, Ficoll-Paque Plus and Ficoll-Paque Premium (Cytiva 17-1440-03 and Cytiva 17-5442-03, respectively, 500mL).
- Use the following volumes of density gradient media per tube capacity:

| **Tube capacity (mL)** | **Density gradient media volume (mL)** |
|---|---|
| 50 | 15 |
| 15 | 6 |

*Follow density gradient media manufacturer's storage recommendations.*

1.2 Filter Sterile Pipette 20µl-1000µl Universal Tips (e.g., Millipore Sigma AXYTF100, 100µl)

1.3 Single Channel Pipette 20µl-1000µl Pipettors (e.g., Pipet-Lite LTS Pipette L-300XLS+, Mettler Toledo, 17014405, 20-300µl)

1.4 Pipet-Aid (cordless preferred) for disposable, serological pipets (e.g., Pipet-Aid Original Portable, 3.3mL/sec, 110 VAC, Drummond, UX-07897-30)

1.5 Serological pipet, disposable, 1, 5, 10, 25, 50mL, sterile (Corning/Costar Stripette polystyrene, individually wrapped, 7536R37, 10mL)

1.6 Centrifuge that meets the following requirements:

- Centrifuge must be capable of generating at least 1500-2000 x g (RCF) at the tube bottom.
- Centrifuge with Swinging Bucket Rotor.
- Tube buckets/adapters for 13 x 100mm and/or 16 x 125mm for selected CPT tube size and 16 - 24 x 50mL conical tubes (CSTFB separation).
- Centrifuge carriers and inserts should be of the size specific to the tubes used.
- Capable of turning off break.

1.7 15 and 50mL disposable centrifuge tubes, sterile, conical bottom, graduated polypropylene (e.g., Nunc 15mL and 50mL conical sterile polypropylene centrifuge tubes bulk, Thermo Scientific, 339650 and 339652, respectively)

1.8 Cell Counting (select and document use of one of the following three options):

- Automated cell counter capable of enumerating viable cells (e.g., Cellometer or equivalent)
- Automated cell counter not capable of distinguishing viable cells (e.g., Coulter Counter, Abbott Cell-Dyn®, Sysmex® or equivalent). Note: An automated cell counter not capable of identifying viable cells may be used to obtain a total cell count without distinguishing viable cells.
- Manual cell counting chamber (hemocytometer) and bright-field microscope.

Note: If a manual cell counting chamber is used with trypan blue, viable cells must be enumerated and used for cell calculations.

1.9 Cell freezing containers that ensure a standardized, controlled rate of -1°C/minute cell freezing in a freezer (-70°C to -95°C). Controlled-rate freezing container options are:

- Mr. Frosty™ by Nalgene, Cat. #5100-0001
- CoolCell® by BioCision, Cat. #BCS-405/BCS-170
- StrataCooler by Agilent, Cat. #400005/400006

1.10 Vials. Options are:

- CoolCell® Filler Vial, 2mL by BioCision, Cat. #BCS-3105 (if using CoolCell® Freezing containers - see above)
- Cryogenic vials (Thermo Scientific™ Nunc Cryogenic vial, Cat. #366656; or Corning

Cryogenic vial, Cat. #430488, or equivalent)

1.11 Class II Biological Safety Cabinet (Certified) – To be used for all steps where the specimen/reagent is uncapped.

1.12 Refrigerator (2°C to 8°C)

1.13 Freezer (-5°C to -20°C)

1.14 Freezer (-70°C to -95°C)

**APPENDIX C: REAGENT PREPARATION**

1.0 Aliquots of FBS-HI (per 8.2.2) that may be needed the next day can be taken out from the freezer and stored at 2°C to 8°C overnight. Mix well before use.

1.1 If PBS is stored at refrigerator temperature (2°C to 8°C), warm up to room temperature (15°C to 30°C) prior to using to wash the cells.

1.2 Prepare and chill freezing media/Cryopreservation Solution (CPS).

1.2.1 Use the following table to calculate the amount of DMSO and FBS needed.

Examples:

| **Estimated freezing media volume** | **DMSO volume = (0.1) (freezing media volume)** | **FBS volume = freezing media volume – DMSO volume** | **Total freezing media volume = DMSO volume + FBS volume** |
|---|---|---|---|
| 10mL | 1mL | 9mL | 10mL |

1.2.2 Mixing of DMSO and FBS is an exothermic reaction. The mixture must be prepared in advance and chilled in the refrigerator (2°C to 8°C) for at least 30 minutes or in an ice bath for at least 15 minutes. After the cooling period and prior to use, the freezing media should be kept at the same temperature as the Freezing Containers outlined in Step 8.10.

1.2.3 The freezing media is stable for 18 hours at 2°C to 8°C after preparation.

1.3 The Freezing Containers (Mr. Frosty/CoolCell/StrataCooler) should be stored per the package insert prior to use.

1.3.1 If using Mr. Frosty: Store at room temperature (15°C to 30°C) prior to use. Isopropanol must be completely replaced after the fifth freeze-thaw cycle. A log sheet should be maintained to accurately keep track of the freeze-thaw cycles and the change of isopropanol after every fifth time.

1.3.2 If using CoolCell: Store at room temperature (15°C to 30°C) prior to use. Insert CoolCell Filler Vials into empty wells of the CoolCell freezing containers when freezing less than a full batch of vials to ensure a consistent freezing rate.

1.3.3 If using StrataCooler: Store at 2°C to 8°C prior to use.